Identification of Estrogenic Tamoxifen Metabolite(s) in Tamoxifen-Resistant Human Breast Tumors

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Purpose: We have shown previously that acquired tamoxifen resistance in an in vivo experimental model is associated with reduced tamoxifen accumulation, isomerization of trans-4-hydroxytamoxifen, and tamoxifen-stimulated tumor growth. The purpose of this study is to isolate and verify the presence of estrogenic tamoxifen metabolites in human breast tumors using high-performance liquid chromatography (HPLC) and mass-spectrometry (MS) techniques.

Patients and Methods: In the present study, we used HPLC and MS to identify the presence of estrogenic metabolites in tumor samples excised from athymic nude mice and in human breast tumors isolated from patients receiving adjuvant tamoxifen therapy.

Results: We identified the presence of metabolite E, a known estrogenic metabolite of tamoxifen, in tamoxifen-resistant MCF-7 human breast tumors implanted in athymic nude mice, as well as in tumors from patients with clinical resistance. Additionally, we separated another estrogenic metabolite, bisphenol, by HPLC, and this was also tentatively confirmed by MS analysis.

Conclusion: These data suggest that cellular tamoxifen metabolism to estrogenic metabolites may in part contribute to stimulating the growth of hormone-responsive breast tumors following prolonged exposure to tamoxifen. Further evaluation of the relationship between cellular metabolism and acquired tamoxifen resistance is warranted.

J Clin Oncol 10:990-994.

TAMOXIFEN IS A nonsteroidal triphenylethylene antiestrogen that is commonly used in the treatment of patients with breast cancer. Tamoxifen competitively antagonizes the binding of estradiol to estrogen receptors, which is thought be the mechanism for inhibiting tumor growth. However, tamoxifen also possesses both estrogenic as well as antiestrogenic effects depending on the tissue and/or species being studied. In some tissues, including estrogen receptor-positive human breast tumors, tamoxifen blocks the growth-stimulating effects of estrogens. However, in other tissues, such as human endometrial tissue, and in some species, such as dogs, tamoxifen acts as an estrogen agonist.[1-3] The mechanisms underlying the "intrinsic" estrogenic effects of tamoxifen remain poorly understood. Because the estrogen agonist-antagonist properties of tamoxifen are highly species- and tissue-dependent, it has been suggested, although it is not yet supported by experimental data, that altered metabolic pathways leading to the production of estrogenic metabolites may be one possible mechanism for the intrinsic estrogenic effects noted with tamoxifen.[3]

The systemic metabolism of tamoxifen is well characterized in humans. The pharmaceutically administered *trans*-tamoxifen is metabolized in the liver to N-desmethyl and 4-hydroxytamoxifen.[4] Both metabolites are active antiestrogenic agents. N-desmethyltamoxifen is the major metabolite found in human serum. It is further metabolized to metabolite Z (didesmethyltamoxifen) and to metabolite Y, the side chain alcohol.[5,6] At least two estrogenic metabolites of tamoxifen have been identified; these include metabolite E and bisphenol.[7] Neither metabolite has been identified in any significant quantity in human serum or tissues. However, metabolite E has been isolated from the bile of dogs, a species in which tamoxifen is predominantly estrogenic.[7] Metabolite E is tamoxifen with a hydroxyl group in place of the dimethylaminoethane side chain, whereas bisphenol is 4-hydroxytamoxifen with a hydroxyl group in place of the dimethylaminoethane side chain.

Two groups have now shown that tamoxifen can actually stimulate tumors to grow following chronic dosing in hormone-responsive human tumors growing in the mouse model.[8-10] We have shown that reduced accumulation and altered metabolism of tamoxifen in the tumor is associated with tamoxifen resistance and tamoxifen-stimulated growth.[10] A similar metabolite profile has been identified in extracts from a panel of human breast cancer specimens from patients with acquired tamoxifen resistance.[9] The possibility that tumor cell growth is dependent on the net estrogen agonist-antagonist activity of intracellular ligands for the estrogen receptor, including tamoxifen metabolites, led us to explore further whether estrogenic metabolites are present in neoplastic breast tissue. In this report, we have now identified the presence of metabolite E in human breast tumor biopsy specimens using high-

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Submitted August 23, 1991; accepted January 21, 1992.

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0732-183X/92/1006-0990$3.00/0

performance liquid chromatography (HPLC) and mass spectrometry (MS) techniques.

PATIENTS AND METHODS

The chemical structures of *trans*- and *cis*-tamoxifen and its metabolites are shown in Fig 1. Tamoxifen citrate (ICI Pharma, Wilmington, DE) and its metabolites *cis*- and *trans*-4-hydroxytamoxifen, bisphenol, and metabolite E were generously provided by Dr John Katzenellenbogen. All reagents were of a HPLC grade including methanol, hexane, 1-butanol (Fisher Scientific, Fair Lawn, NJ), and triethylamine (Sigma Chemical Co, St Louis, MO).

Human Breast Tumor Specimens

Five human breast tumors from the San Antonio Breast Tumor Bank that contained sufficient tissue for assay were provided by Dr William McGuire. Four of the patients demonstrated disease progression on tamoxifen (10 mg twice per day) after treatment periods ranging from 2 months to 5 years. Tissue was obtained from one patient who had been on tamoxifen for only 1 month. This patient developed disease progression 1 month later. Three of these patients had de novo tamoxifen resistance and two had acquired resistance after an initial response. A portion of each tumor was extracted, irradiated, and analyzed by HPLC, while the remaining portion was extracted, fractionated, and analyzed by MS (see HPLC and MS Analysis).

MCF-7 Breast Tumor Specimens

Tumor tissue was also obtained from an in vivo nude mouse model of acquired tamoxifen resistance. MCF-7 human breast tumors were harvested from nude mice after the development of tamoxifen resistance (4 to 6 months of intraperitoneal tamoxifen injections, 5/0 μg/d). These tissues were frozen at −20°C until analysis.

HPLC and MS Analysis

Frozen tissue samples were weighed, homogenized, and extracted according to previous methods[10,11]. Briefly, all samples were extracted with 6.5 mL of 2% butanol in hexane, vortexed for 1 minute, and then centrifuged for 10 minutes at 1,500 rpm. The organic phase was dried under nitrogen gas at 37°C and reconstituted in 200 μL methanol before injection. The reconstituted samples were transferred to an Infrasil quartz cuvette (Fisher Scientific, Fair Lawn, NJ), irradiated for 1 minute with a 15 W mercury vapor lamp, and injected onto the column.

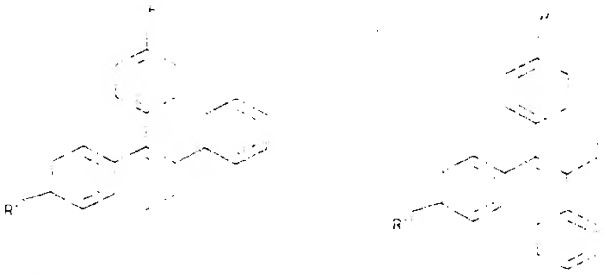

Fig 1. Structures of trans- and cis-tamoxifen isomers. *Trans*-tamoxifen, R is $-OCH_2CH_2N(CH_3)_2$; R′ is −H, 4-hydroxytamoxifen, R is $-OCH_2CH_2N(CH_3)_2$; R′ is −OH, N-desmethyltamoxifen; R is $-OCH_2CH_2NHCH_3$; R′ is −H, bisphenol; R is −OH; R′ is −H, monophenol; R is −OH; R′ is −H, metabolite Y; R is $-CH_2CH_2OH$; R′ is −H, metabolite Z; R is $-OCH_2CH_2NH_2$; R′ is −H, metabolite Bx; R is $-OCH_2CH_2NHCH_2$; R′ is −OH. *Cis*-tamoxifen; R is $-OCH_2CH_2N(CH_3)_2$; R′ is −H, 4-hydroxytamoxifen; and R is $-OCH_2CH_2NHCH_3$; R′ is −OH.

The HPLC system consisted of a Beckman model 32[1] gradient liquid chromatograph (Fullerton, CA), two model 110A pumps, and a model 420 controller. The HPLC was equipped with a reverse-phase Altex Ultrasphere column (Alameda, CA) and a 100 μL injection loop. The mobile phase consisted of 5% water and 0.1% triethylamine in methanol. The flow rate of the mobile phase was set at 0.5 mL/min. The fluorescence of photochemically activated compounds was detected with a Applied Biosystems 980 fluorometer with excitation wavelength set at 296 nm. Retention times and peak heights were recorded with a Spectraphysics 4290 integrator (Piscataway, NJ).

Nonirradiated samples were also injected onto the HPLC column and fractions were collected every minute. Fractions corresponding to the retention times noted for bisphenol and metabolite E were further evaluated using MS. The MS operation of these metabolites under electron impact ionization MS (EI-MS) was used. HPLC-generated samples in glass vials were extracted with methanol, injected into glass capillaries, and dried. Samples were introduced via a direct insertion probe. Spectra were recorded at 70 eV over the mass range of 50 to 600 at 10 seconds per decade with a resolution of 1,000. The probe was heated in stages to 250°C.

RESULTS

Human Tumor Specimens

All five specimens from patients with tamoxifen resistance had peaks on HPLC that comigrated with stock solutions of metabolite E. HPLC analysis of a representative human breast cancer tissue specimen and a spiked sample is shown in Fig 2. In this human breast cancer biopsy specimen, peaks corresponding to the retention times of bisphenol, metabolite E, *cis*-4-hydroxy, *trans*-4-hydroxy, metabolite Bx, tamoxifen, metabolite Z, and N-desmethyltamoxifen were observed. Tamoxifen and its N-desmethyl metabolite were the most abundant compounds present (200 to 400 ng/g). Peaks were also noted for metabolites Bx and Z; however, these were not quantified. Three of the five specimens had peaks comigrating with a bisphenol standard. Peaks corresponding to bisphenol, metabolite E, *cis*-4-hydroxy, metabolite Y, and *trans*-4-hydroxy tamoxifen were present in low concentrations (less than 50 ng/g) but because of the close proximity of their retention times, absolute identification of these peaks required MS analysis.

Further confirmation of the bisphenol and metabolite E HPLC fractionations were attempted using ME-MS analysis confirmed the presence of metabolite E in two specimens, one of which is shown in Fig 3A. The HPLC peak at 11 to 12 minutes corresponded to a peak at 300 m/z, consistent with the molecular weight of metabolite E. In addition to the peak at 300 m/z, metabolite E has

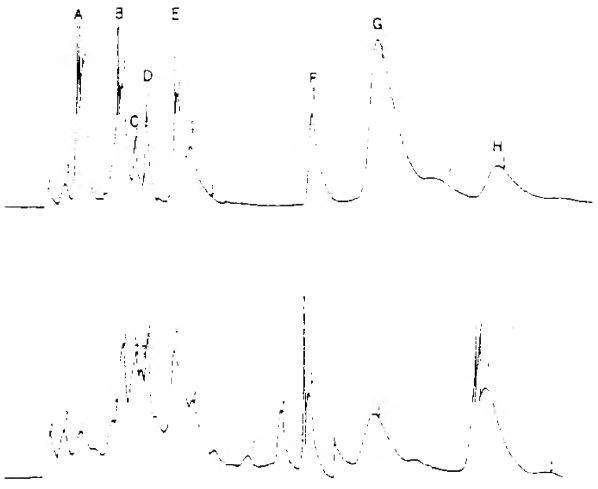

Fig 2. Separation of tamoxifen and metabolites in a spiked plasma sample (top) and in a tamoxifen-resistant human breast tumor (bottom). Peaks are bisphenol (A), metabolite E (B), *cis*-4-hydroxytamoxifen (C), *trans*-4-hydroxytamoxifen (D), metabolite Bx (E), tamoxifen (F), metabolite Z (G), and N-desmethyltamoxifen (H).

strong peak at 149 m/z was observed. This was probably derived from phthlate ester impurities in the sample. Although a small but clear peak comigrates with bisphenol on HPLC, the relative abundances of smaller weight compounds (< 150) obscured the MS analysis of these peaks. Therefore, the presence of bisphenol could not be confirmed in this specimen. One specimen did have a clearly identified bisphenol peak at 316 m/z (Fig 3B). However, this result must be considered preliminary because there was insufficient material to confirm the result. Neither metabolite E nor bisphenol could be confirmed by MS in the other specimens because of insufficient material.

Tamoxifen-Resistant MCF-7 Tumors

Results from HPLC analysis of an MCF-7 tumor isolated from a nude mouse following prolonged tamoxifen administration are shown in Fig 4. The top chromatogram shows results of the MCF-7 tumor analysis whereas the bottom shows a plasma sample spiked with bisphenol (A), metabolite E (B), *cis*-4-hydroxy (C), *trans*-4-hydroxy (D), tamoxifen (E), and N-desmethyltamoxifen (F). Peaks with retention times corresponding to metabolite B, *cis*-4-hydroxy, *trans*-4-hydroxy, tamoxifen, and N-desmethyltamoxifen were noted in the MCF-7 tamoxifen-resistant tumor. Metabolite BX was also noted on the chromatogram (retention time, 17 to 19 minutes), but it was not quantified. N-desmethylation and hydroxylation were the primary metabolic routes noted. Concentrations of tamoxifen and its metabolites were as follows: tamoxifen 3,255.9 ng/gm; N-desmethyltamoxifen 135.4 ng/gm; *cis*-4-hydroxytamoxifen 42 ng/gm; and *trans*-4-hydroxytamoxifen 36 ng/gm. Similar to the results noted in the human tumor specimen, the MCF-7 tumor also had peaks corresponding to that of metabolite E (22 ng/g). A small peak was evident at a retention time similar to that of bisphenol. In several specimens these peaks were confirmed as metabolite E by MS (Fig 3C).

DISCUSSION

In patients receiving tamoxifen therapy, de novo resistance is common, and acquired resistance to tamoxifen invariably occurs in all patients. Several mechanisms of resistance have been suggested including the selection of a hormone-independent clone and altered metabolic pathways.[3] In the present study, we have documented, by both HPLC and MS, that an estrogenic

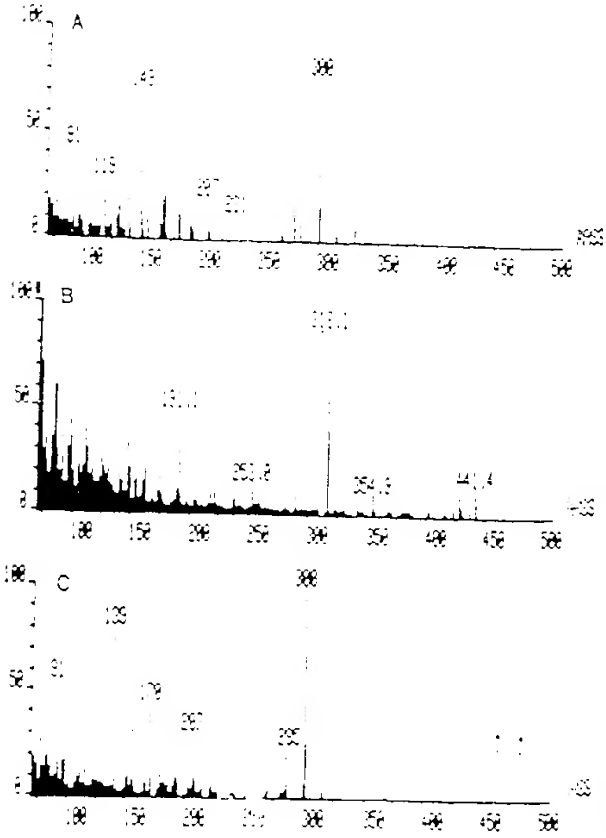

Fig 3. Electron-ionization(+)MS result of peaks isolated by HPLC fractionation of tamoxifen-resistant human breast tumors (A and B) and MCF-7 tamoxifen-resistant tumor (C). The peak at 300 MS confirms the presence of metabolite E tamoxifen (A and C), whereas a 316 MS corresponds to bisphenol.

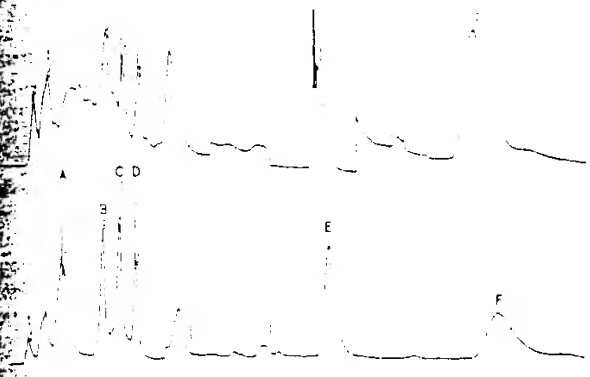

Fig 4. HPLC chromatograms showing tamoxifen and metabolites in (top) MCF-7 tamoxifen-resistant tumor grown in a nude mouse following tamoxifen administration (500 μg/d times 6 months) and (bottom) plasma sample spiked with bisphenol (A), metabolite E (B), *cis*-4-hydroxytamoxifen (C), *trans*-4-hydroxytamoxifen (D), tamoxifen (E), and N-desmethyltamoxifen (F).

metabolite (metabolite E) is present in tumors isolated from patients who have undergone unsuccessful tamoxifen therapy even after relatively short treatment durations. Additionally, we observed that bisphenol, a potent estrogenic metabolite, was also present, although this could not be definitely confirmed by MS. These metabolites were detected in tamoxifen-resistant human breast tumor tissue, as well as in resistant MCF-7 tumors isolated from nude mice treated with tamoxifen. In preliminary studies we have also identified metabolite E in tamoxifen-sensitive MCF-7 tumors but at a much lower concentration relative to the parent drug and other antiestrogen metabolites.

The presence of other metabolites was also noted in both mouse and human tumors. The antiestrogenic metabolites 4-hydroxytamoxifen and N-desmethyltamoxifen were the primary metabolites noted. Metabolite Bx was also identified in tumors. This metabolite binds to estrogen receptors with higher affinity than tamoxifen. however, its antiestrogenic activity has not been fully elucidated.[12] Metabolite Bx has been identified in the human serum and is present in high concentrations in bile and urine following chronic dosing.[13]

The identification of estrogenic metabolites in tissues following long-term tamoxifen administration has important clinical ramifications. Both bisphenol and metabolite E have documented estrogenic activity in vitro and in vivo. Metabolite E can exist in either the *cis*- or *trans*-chemical configuration. Isomerization of *trans*-metabolite E to *cis*-metabolite E has been demonstrated in vitro.[14] The structure activity relationship of these isomers has also been examined. *Trans*-metabolite E seems to be a partial agonist, whereas the *cis*-isomer is a potent estrogen agonist. The *cis*-form of metabolite E has also been shown to be more potent than bisphenol in stimulating T47D human breast cell growth.[14]

Metabolite E has been identified in laboratory animals, but no conclusive evidence exists establishing its presence in human tumors. Using gas-chromatography MS (GC-MS), Fromson et al identified metabolite E in the bile fluid of dogs in which it accounted for approximately 6% of the radiolabeled compounds present in bile.[15] Murphy et al tentatively identified the presence of *cis*-metabolite E using GC-MS in the plasma of patients receiving tamoxifen.[16] They report that *cis*-metabolite E was present in low concentrations (0.9 to 2.0 ng/mL) in patients given tamoxifen for 14 days, whereas concentrations of 2.8 and 7.0 ng/mL were reported in two chronically treated patients (> 2 years).[16]

Our previous studies in an experimental model and in tumors from patients suggest that acquired tamoxifen resistance is associated with reduced tamoxifen concentrations and increasing *cis*- to *trans*-4-hydroxytamoxifen ratios in the tumor itself.[10,11] Furthermore, tamoxifen resistant tumors in this model are actually being stimulated to grow by tamoxifen.[10] The identification of the estrogenic metabolite (metabolite E) in tamoxifen-resistant human breast tumors represents an additional clue as to the mechanism of acquired tamoxifen resistance and of tamoxifen-stimulated growth. Reduced accumulation of antiestrogenic compounds (*trans*-tamoxifen, 4-hydroxytamoxifen, and N-desmethyltamoxifen) coupled with a relative accumulation of estrogenic metabolites, such as metabolite E or bisphenol, may result in a net estrogenic environment within normonail responsive breast tumor, favoring stimulation rather than inhibition of tumor growth.

Further evidence of tamoxifen-stimulated growth is observed in patients who are progressing on tamoxifen after an initial response and then respond again to its withdrawal.[6] Tamoxifen-stimulated growth may also explain the failure of patients to respond to ovarian ablation after progressing on tamoxifen only when tamoxifen is not discontinued at the time of castration.[17,18] Additional studies are needed to confirm whether estrogenic metabolites are contributing to the development of tamoxifen resistance.

ACKNOWLEDGMENT

We would like to dedicate this report to the memory of Dr William L McGuire. We also thank Walter McMurray for his expertise in mass-spectrometric analysis, which was performed at the Yale Comprehensive Cancer Center, Yale University.

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